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(54) Title: ENHANCEMENT OF IMMUNE RESPONSE USING TARGETING MOLECULES

(57) Abstract

The present invention provides methods of enhancing the immune response to an immunogen and to compositions for use in these methods. In particular the present invention provides a DNA molecule for use in raising an immune response to an antigen. The DNA molecule includes a first sequence encoding a targeting molecule, a second sequence encoding the antigen or an epitope thereof, and optionally a third sequence encoding a polypeptide which promotes dimerisation or multimerisation of the product encoded by the DNA molecule.

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Enhancement of Immune Response Using Targeting Molecules

FIELD OF THE INVENTION

The present invention relates to methods of enhancing the immune response to an immunogen and to compositions for use in these methods. In particular the present invention relates to the use of targeting molecules in DNA and protein vaccination.

BACKGROUND OF THE INVENTION

The ability of direct injection of non-replicating plasmid DNA coding for viral proteins to elicit protective immune responses in laboratory and preclinical models has created increasing interest in DNA immunisation. A useful review of DNA vaccination is provided in Donnelly *et al*, Journal of Immunological Methods 176 (1994) 145-152, the disclosure of which is incorporated herein by reference.

Intramuscular injection of DNA as a means of vaccination can induce both cellular and humoral responses (1). Studies using reporter proteins demonstrated that muscle cells are the principal target for transfection after intramuscular DNA injection (2). The mechanisms underlying the induction of immune responses after DNA immunisation are unclear. Since myocytes express MHC Class I at low levels and do not constitutively express Class II or costimulatory molecules such as B-7 (3), they appear unlikely candidates for the induction of Ab or CTL responses. It is possible that low level transfection of antigen presenting cells (APCs) occurs at the injection site and these APCs then traffic to lymphoid organs and present the encoded antigen to B and T cells (4) as has been shown after intradermal (5) and biolistic DNA immunisation (6). Alternatively the myocyte may act merely as a source of antigen and priming occurs in the draining lymph node. In the latter case, optimum immune induction would result if the antigen was released from the myocyte by secretion or subsequent to cell damage.

One strategy that has been shown to augment the response to polynucleotide, or DNA, vaccination is the use of sequences encoding cytokines or co-stimulatory molecules (Conry *et al*, (1996) Gene Therapy

3: 67-74). These investigators showed an increased response when the DNA administered encoded not only the antigen of interest but also for B7-1.

The present inventors investigated the effects of modifying the antigen such that it will be targeted to APC or sites of immune induction. This was shown to not only markedly enhance the immune response but also cause immune deviation.

SUMMARY OF THE INVENTION

In a first aspect the present invention consists in a DNA molecule for use in raising an immune response to an antigen, the DNA molecule including a first sequence encoding a targeting molecule, a second sequence encoding the antigen or an epitope thereof, and optionally a third sequence encoding a polypeptide which promotes dimerisation or multimerisation of the encoded product.

As will be appreciated by those skilled in the art in a number of instances the antigen or epitope encoded by the second sequence will be a polypeptide which promotes dimerisation or multimerisation of the encoded product. As will be understood in such instances the third sequence may be omitted.

In a second aspect the present invention consists in a polypeptide, the polypeptide being encoded by the DNA molecule of the first aspect of the invention.

In a third aspect the present invention consists in a method of raising an immune response in an individual, the method comprising administering to the individual the DNA molecule of the first aspect of the present invention or the polypeptide of the second aspect of the present invention.

There are a wide range of molecules which could be used as targeting molecules. These include ligands which target lymphoid cells (which will either be at or take the Ag to sites of immune induction), lymphoid sites (eg. spleen, lymph nodes, Peyers patches) or APCs directly. Examples of such ligands include, but are not limited to, CD40L, OX40, antibodies to receptors on APCs (eg. DEC 205, CD 23, CD11c, MHC class II), CD28, CTLA4 and L-selectin. It is presently preferred that the targeting molecule is CTLA4 or L-selectin.

In a fourth aspect the present invention consists in a method of deviating the immune response to an antigen in an individual, the method comprising administering to the individual a DNA molecule including a first sequence encoding CTLA4, a second sequence encoding the antigen or an epitope thereof, and optionally a third sequence encoding a polypeptide which promotes dimerisation or multimerisation of the encoded product.

There are many ways of producing dimerisation or multimerisation including tandem duplication and the use of any molecule that normally forms multimers (e.g. Immunoglobulins, CD8, TNF, glutathione s-transferase, zinc finger dimers etc). There are many references in the scientific literature regarding this area. These include Classon BJ et al (1992) "The hinge region of the CD8 alpha chain: structure, antigenicity, and utility in expression of immunoglobulin superfamily domains" Int Immunol 4:215-25; Yang J, Moyana T, Xiang J (1995) "A genetically engineered single-chain FV/TNF molecule possesses the anti-tumor immunoreactivity of FV as well as the cytotoxic activity of tumor necrosis factor." Mol Immunol. 32:873-81; Tudyka T, Skerra A (1997) "Glutathione s-transferase can be used as a c-terminal, enzymatically active dimerization module for a recombinant protease inhibitor, and functionally secreted into the periplasm of *Escherichia coli*." Protein Science. 6:2180-2187; Pomerantz JL, Wolfe SA, Pabo CO (1998) "Structure-based design of a dimeric zinc finger protein" Biochemistry 37:965-970; and Whiteheart SW, Rossnagel K, Buhrow SA, Brunner M, Jaenicke R, Rothman JE (1994) "N-ethylmaleimide-sensitive fusion protein: a trimeric ATPase whose hydrolysis of ATP is required for membrane fusion." J Cell Biol 126:945-54. The disclosure of these references and the other references referred to in this application are included herein by cross-reference.

As will be appreciated by those skilled in the art in the constructs of the present invention the first, second and third DNA sequences may be in any particular order. It is presently preferred that the order is first, third then second.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

DETAILED DESCRIPTION OF THE INVENTION

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following examples and Figures in which:

Figure 1. Secretion of ΔIg, CTLA4Ig and L-SELIg proteins from NIT transfectants. NIT cells were transfected with the pRep10::CD5L-hIg, pRep7::mCTLA4-hIg and pRep10::hL-SEL-hIg expression plasmids. Secreted protein was purified on immobilised protein A and samples run by SDS PAGE under reducing and non-reducing conditions.

Figure 2. hIg specific IgG responses in DNA immunized mice. Sera were obtained from BALB/c mice immunized with pRep10::CD5L-hIg, pRep7::mCTLA4-hIg and pRep10::hL-SEL-hIg at the indicated times post immunisation and stored at -20°C until assayed for hIg specific IgG in an ELISA. Titres were defined as the highest dilution to give a 0.2 OD at 450 nm. Results are expressed as the mean of the log titre ±SEM from 5 mice in each group. Normal mouse sera and hyperimmune mouse sera served as the negative and positive controls respectively.

Figure 3. hIg specific IgG subclass responses in DNA immunized mice. A. Sera were obtained from BALB/c mice immunized with pRep10::CD5L-hIg, pRep7::mCTLA4-hIg and pRep10::hL-SEL-hIg at 8 weeks post immunisation and stored at -20°C until assayed for hIg specific IgG1, IgG2a or IgG2b in an ELISA. Titres were defined as the highest dilution to reach an OD of 0.2 at 450 nm. Results are expressed as the mean of the log titre ±SEM from 5 mice in each group. B. The log IgG1 titre for each mouse was divided by the corresponding log IgG2a titre to obtain a log IgG1: log IgG2a ratio. Results are expressed as the mean ±SEM from 5 mice in each group.

Figure 4. hIg specific IgG subclass responses in soluble protein immunized mice. Sera were obtained from BALB/c mice immunized with 5 μg of hIg or 5 μg of CTLA4Ig protein in 100 μl of PBS, 2 weeks post immunisation and assayed for hIg specific IgG in an ELISA. Titres were defined as the highest dilution to reach an OD of 0.2 at 450 nm. Results are expressed as the mean of the log titre ±SEM from 5 mice in each group.

Figure 5. hIg specific IgG subclass responses in CTLA4Ig DNA immunized mice. Sera were obtained from BALB/c mice immunized with the indicated dose of pRep7::mCTLA4-hIg 2 weeks post immunisation and assayed for hIg specific IgG1, IgG2a or IgG2b in an ELISA. Titres were defined as the highest dilution to reach an OD of 0.2 at 450 nm. Results are expressed as the mean of the log titre ±SEM from 5 mice in each group.

Figure 6. OVA specific IgG and IgG subclass responses after co-injection of DNA. Sera were obtained from BALB/c mice immunized with pRep10::hL-SEL-hIg and pCI-OVA or pRep7::mCTLA4-hIg and pCI-OVA at 4 weeks post immunisation and assayed for OVA specific IgG (A) or IgG1, IgG2a or IgG2b (B) in an ELISA. Titres were defined as the highest dilution to reach an OD of 0.2 at 450 nm. Results are expressed as the mean of the log titre ± SEM from 5 mice in each group.

Figure 7. Shows stimulation index with hIg, Lsel-hIg and CTLA4-hIg (▒ HuIg 1mg/ml; ▒ HuIg 1mg/ml; ▒ HuIg 1mg/ml)

Figure 8. Shows anti-ovalbumin IgG titres with various constructs (▒ 2 weeks; ▒ 4 weeks)

Figure 9. Shows anti-OVA IgG titres with pCI::mCTLA4-g3h-OVA and pCI::mCTLA4-hIg-OVA

Figure 10. Groups of 5 Balb/c mice were vaccinated intramuscularly on days 0 and 28 with 0.1mg of pCI::mCTLA4-hIg-45W (black circles) or pCI::CD5L-hIg-45W (grey circles). Mice were bled on days 0, 7, 14, 28, 35 and 42. Sera was assayed for anti-45w antibodies by ELISA using recombinant 45W(His)6. The Student's t-test was used to compared the two groups and the probability values (P) for the two vaccines at each time point are shown at the top of the figure.

Figure 11. Groups of 5 Balb/c mice were vaccinated either intraperitoneally with 20μg of recombinant 45w(His)6 protein (grey circles) in Freund's complete adjuvant or intramuscularly with 0.1mg pCI::mCTLA4-hIg-45W (black circles) in 0.1ml of saline. Mice were bled on days 0, 2 ,5 ,8 , 14 and 28 post-vaccination and anti-45w antibodies measured by ELISA using 45W(His)6 protein. The responses were compared by Student's t-test and were different at day 8 ($p<0.05$).

Figure 12. Shows % survival of mice following challenge with *Plasmodium chabaudi adami* DS (▒ pCI::CD5LhIg-AMA; ▒ pCI::CTLA4-hIg-AMA; ▒ pCI::CTLA4-hIg).

Figure 13 shows antibody titres.

Figure 14 shows antibody titres at day 14 with varying immunisations

Figure 15 shows antibody titres at day 54 with varying immunisations

Figure 16 shows lung virus titres

Figure 17. Kinetics of induction of the anti-hIg antibody titres post-immunisation. Filled symbols represent plasmids containing hIg: filled squares (pCI::bCTLA4-hIg-ΔPLD), filled circles (pCI::CD5L-hIg-ΔPLD), filled triangles (pCI::bCTLA4-hIg). Open symbols represent control animal groups not injected with hIg in any form: open circles (pCI::ΔPLD), open squares (Unvaccinated controls) and open triangles (Glan-Vac).

Figure 18. Western Blot of PLD expressed by eukaryotic and prokaryotic cells. Lane 1-3. Supernatant from Cos-m6 cells transfected with pCI::PLD (lane 1), pCI::ΔPLD (lane 2) and pCI alone (lane 3). Lane 4. Cell filtrate containing PLD expressed from *Corynebacterium pseudotuberculosis*

Figure 19. Protection from challenge with *Corynebacterium pseudotuberculosis*. Percentage of the animals protected from challenge by 10^6 CFU of *Corynebacterium pseudotuberculosis* injected just above the coronet. Protection was defined as the animal not having abscesses in any of the following lymph nodes: popliteal, inguinal and prefemoral both left and right.

Figure 20 Shows number of mice with lesions at various time points after challenge with *L. major* (□ PBS; ▲ pCI::CD5L-hIg-PSA2; ■ pCI::mCTLA4-hIg-PSA2; Δ pCI::PSA2)

EXAMPLE 1

Materials and Methods

Mice

Female mice (BALB/c, CBA and C57Bl/6) aged 6 to 8 weeks were used in all experiments. Mice were maintained in SPF conditions.

Plasmids and immunisations

Expression plasmids were constructed to produce secreted forms of the Fc fragment of human IgG1 (ΔIg) by using the Cd5 leader sequence (CD5L) either alone or fused with murine CTLA4 (mCTLA4Ig) or human

L-selectin (hL-SELIg) under the control of the RSV promoter in the Rep7 or Rep10 vectors (these vectors differ only in the direction of the multiple cloning site, Invitrogen, San Diego, CA, USA). The sequence of pREP7::CTLA4-hIg is shown in Sequence ID No. 1 - Promoter RSV: 13-640, CTLA4-hIg: 703-2462. The constructs were obtained from plasmids given by Drs. P. Lane (Basel Institute, Switzerland), B. Seed (Massachusetts General Hospital, Boston, USA) and D. L. Simmons (Institute of Molecular Medicine, Oxford, UK). The following constructs were generated:

pRep10::CD5T-hIG
pRep7::mCTLA4-hIg
pRep10::hLSEL-hIg

Plasmids for injection were prepared from *E. coli* by PEG precipitation as described (7) except that volumes of Solution I, II and III were adjusted such that pellets were resuspended in 50 mL of Solution I for each Litre of broth media used. Endotoxin was removed from plasmid preparations by four Triton X-114 phase separations (8) and DNA was stored at -20°C in normal saline until injected. The resultant plasmid preparations contained less than 10 IU endotoxin per mg of plasmid DNA as determined by the limulus amoebocyte lysate assay (QCL -1000 BioWhittaker, Walkersville, MD, USA). Mice received 100 µg of plasmid DNA in both quadriceps or intradermally at the base of the tail on day 0 and 14 of each experiment.

Antibody assays

Microtitre plates (Dynatech, Chantilly, VI, USA) were coated with human Ig (hIg) protein (Intragam, CSL, Parkville, Australia; 10 µg/ml in PBS) by overnight incubation at 4°C and washed four times with PBS to remove unbound antigen. Plates were incubated with serially diluted sera in blocking buffer (5% milk powder in PBS) overnight at 4°C. After washing 5 times with PBS to remove unbound Ab, plates were incubated with peroxidase conjugated anti-mouse IgG, IgG1, IgG2a or IgG2b antibodies (Southern Biotechnology, Birmingham, AL, USA) diluted in blocking buffer. After washing five times with PBS, the amount of bound Ab was determined by addition of substrate solution (0.1 mg/ml 3,3,5,5-tetra methylbenzidine (T2885, Sigma St. Louis, MO, USA) 0.03% H_2O_2 in 0.1M Na acetate pH 6.0). The reaction was stopped with 1M H_2SO_4 and the OD read at 450nm. Titres were defined as the highest dilution to reach an OD of 0.2.

To calibrate the IgG subclass ELISA, plates were coated with IgG1, IgG2a or IgG2b from mouse myelomas (10 μg/ml in 0.5 times PBS) overnight at 4°C, washed 3 times with PBS and then incubated with serially diluted anti-mouse IgG subclass HRP conjugated Ab. The dilution of each anti-mouse subclass Ab which gave identical absorbances in the ELISA were used subsequently.

Results and Discussion

Expression plasmids were constructed to produce secreted forms of the human IgG1 heavy chain alone (pRep10::CD5L-hIg) or fused with CTLA4 (pRep7::mCTLA4-hIg) or L-selectin (pRep10::hLSEL-hIg). Cells transfected with these plasmids secreted the three molecules as disulphide linked dimers of expected size (Fig. 1). Like others (1) we were unable to detect *in vivo* protein expression by western blotting of muscle homogenates and or of protein A purified material from sera of B-cell deficient immunized mice (data not shown). However, the ability to detect immune responses in immunized mice is indicative of *in vivo* expression. No immune responses to human Ig were detected in unimmunised or mice receiving vector only (data not shown). However, mice immunized with pRep10::CD5L-hIg, pRep10::hL-SEL-hIgΔIg or pRep7::mCTLA4-hIg had markedly different responses (Fig. 2). Responses in pRep7::mCTLA4-hIg immunized mice were more rapid and of greater magnitude at all three time points: 2, 4 and 8 weeks (Fig. 2). At 4 weeks both the pRep7::mCTLA4-hIg and pRep10::hLSEL-hIg immunized mice had 1000 and 100 fold higher IgG responses than pRep10::CD5L-hIg controls respectively. The differences observed were not attributable to any adjuvant effects of endotoxin, because Triton X-114 was used to remove endotoxin (8) so that the levels were <10IU/mg plasmid DNA. Similar results have been achieved in 3 experiments using BALB/c and CBA mice (data not shown).

The response in all mice to pRep10::CD5L-hIg was dominated by IgG2a (Fig. 3), which mimics a viral infection, and has been reported for other antigens after DNA immunisation (9, 10). The IgG subclass response in pRep10::hLSEL-hIg immunized mice was similar (although greater) to pRep10::CD5L-hIg controls whereas the pRep7::mCTLA4-hIg response was deviated to an IgG1 dominance (Fig. 3B). The possibility that the differences

in Ab responses was due to dose was unlikely since all constructs were made with identical plasmid backbone and mice immunized with soluble CTLA4Ig protein (Fig. 4) had higher Ab responses than those receiving an equivalent dose of hIg. Also, to determine if the IgG1 dominance of the response to pRep7::mCTLA4-hIg was due to dose we immunized mice with different amounts of pRep7::mCTLA4-hIg so that mice with total IgG antibody levels could be compared to that of pRep10::hLSEL-hIg (Figs. 2 and 3). The IgG1 predominance was found at all doses of pRep::mCTLA4-hIg (Fig. 5).

Work with CTLA4 has demonstrated it can bind to B-7 and block co-stimulation which reduces the response to other immunogens (11). A non-specific immunomodulatory effect of CTLA4 was unlikely for several reasons. Firstly, CTLA4Ig protein at least in high doses (and hence B-7 is blocked) has been ascribed immunosuppressive properties not immunostimulating ones (11) as we found for DNA and protein immunisations. Furthermore, mice co-injected with CTLA4Ig and DNA encoding ovalbumin (pCI-OVA) had similar ovalbumin specific IgG and IgG subclass titres to control mice (Fig. 6) indicating that there was not any immunosuppressive effect of CTLA4.

Example 2

Use of targeting ligand to augment T cell proliferative responses and requirement for dimerisation

Introduction

In Example 1 there is a demonstration that Ab levels to a model DNA vaccine could be enhanced when antigen was fused with the targeting ligands CTLA4 or L-selectin.

The hIg component would ensure dimerisation which we thought would be favourable because in general, binding of ligands to receptors is stronger when dimers are used. However, it was unclear in this system if dimerisation of the antigen targeting ligand fusion proteins was necessary for increased immune responses. To determine if the enhanced Ab response generated by antigen targeting vectors encoding proteins was dependent upon dimer formation, Ab responses were compared to immunisation with plasmid encoding monomeric antigen targeting ligand fusion proteins. The

hIg component of the vectors was replaced with coding sequence for another model antigen that would not form dimers (ovalbumin; OVA).

Materials and Methods

Female mice aged 6 to 8 weeks were used in all experiments and maintained in SPF conditions.

After PCR amplification to include an Mlu I restriction enzyme recognition sequence, the OVA cDNA (bp 470-1170) was inserted behind the human immunoglobulin Fc (hIg) gene via a 4 amino acid glycine linker at the Nsi I site. These vectors would form dimers due to the interchain disulfide bonds of hIg and are represented by an hIg-OVA suffix. A targeting vector that would not form dimers was obtained by direct fusion of the cDNA from OVA to the cDNA of CTLA4 (pCI::mCTLA4-OVA) or to the leader sequence of CD5 as a control (pCI::CD5L-OVA). After PCR amplification to include Hind III and Nsi I restriction sites the entire hIg component of pCI::mCTLA4-hIg-OVA was replaced with the human IgG3 hinge region (a gift from Dr Y Akahori, Japan) to form pCI::mCTLA4-g3h-OVA. Plasmids for injection were prepared from *E. coli* with endofree QIAGEN maxi kits according to the manufacturer's instructions and stored at -20°C in normal saline until injected. Mice received 50 µg of plasmid DNA in 100 µl normal saline i.m. in both quadriceps at day 0 of each experiment.

The proliferation of 2 X 10^5 splenocytes was determined by a standard 5 day ^{3}H-thymidine uptake protocol at 6 weeks post initial immunisation. The mean stimulation index was calculated as the cpm with antigen / cpm splenocytes alone.

Microtitre plates (NUNC, Maxisorb) were coated with OVA protein (A-5503, Sigma, St. Louis, MO; 10 µg/ml in PBS) by overnight incubation at 4°C and washed four times with PBS to remove unbound antigen. Plates were incubated with serially diluted sera in blocking buffer (1% casein in PBS) overnight at 4°C. After washing 5 times with PBS to remove unbound Ab, plates were incubated with peroxidase conjugated anti-mouse IgG (Southern Biotechnology, Birmingham, AL) diluted in blocking buffer. After washing five times with PBS, the amount of bound Ab was determined by addition of tetramethylbenzidine substrate solution. The reaction was

stopped with 1M H_2SO_4 and the OD read at 450nm. Titres were defined as the highest dilution to reach an OD of 0.2.

Results and Discussion

The proliferation splenocytes was determined by a standard 5 day 3H-thymidine uptake protocol at 6 weeks post initial immunisation (Fig. 7). The stimulation index was calculated as the cpm with antigen / cpm splenocytes alone. The mean ±SD from 3 mice in each group is shown after incubation with three different antigen concentrations. Mice immunized with the DNA constructs pCI::mCTLA4-hIg and pCI::Lsel-hIg had 8 and 3 fold higher T cell proliferative responses than controls (pCI::CD5L-hIg) respectively . This data suggested that the targeting of antigen was having an enhancing effect on T cell activation.

Groups of 8 mice were immunized with DNAs expressing the monomeric targeting vector pCI::mCTLA4-OVA, the monomeric control pCI::CD5L-OVA, or the dimeric vectors pCI::CD5L-hIg-OVA (control), pCI::Lsel-hIg-OVA or pCI::CTLA4-hIg-OVA on day 0 and bled 2 and 4 weeks post immunisation. The OVA specific IgG levels were determined by ELISA. The results obtained at 2 and 4 weeks post immunisation are illustrated in Fig. 8 (2 weeks; hatched columns, 4 weeks; solid columns). There was no difference in Ab levels at 2 or 4 weeks between the mice immunized with pCI::CD5L-OVA or pCI::mCTLA4-OVA monomeric DNA vectors. The highest Ab responses were obtained with the pCI::mCTLA4-hIg-OVA vector, which forms dimers, compared to the monomeric (pCI::CD5L-OVA) or dimeric (pCI::CD5L-hIg-OVA) controls.

Surprisingly, the pCI::Lsel-hIg-OVA immunized mice had the poorest responses at both time points. This data is in contrast to the enhanced responses to hIg when fused with L-selectin alone. The observation that the responses obtained with pCI::Lsel-hIg-OVA were similar in magnitude to those obtained with the monomeric antigen fusions suggests that the fusion of OVA (or other antigens) to Lsel-hIg may interfere with the efficiency of binding of L-selectin to its ligand (e.g. by interfering with dimerisation, by allosteric effects or by conformational changes to L-selectin). Alternative ways of fusion should be investigated.

Overall, these results suggest that for effective antigen targeting the inclusion of a molecule such as hIg that facilitates dimerisation is essential unless the antigen itself facilitates dimerisation or multimerisation. This data was obtained using the hinge, CH2 and CH3 domains of human IgG1. The dimerisation of hIg is facilitated by disulfide bonds between cysteine residues in the hinge domains. To determine if another molecule that would also facilitate dimerisation could replace the hIg component, the hinge region of human IgG3 was used to link mCTLA4 with OVA. Groups of 8 mice were immunized with DNAs expressing the targeting vectors pCI::mCTLA4-hIg-OVA or pCI::mCTLA4-g3h-OVA. At 2 weeks post immunisation sera was collected and shown to contain similar levels of anti-OVA antibodies (Fig. 9). Therefore, this suggests that it may be possible to reduce the hIg component of the antigen targeting vectors to a hinge region alone or replace the hIgG1 component with another immunoglobulin hinge region, another molecule or part thereof such as the hIgG3 hinge to facilitate dimerisation. This would be of particular applicability when the targeting ligand is L-selectin or another molecule that does not dimerise or is structurally compromised by fusion of antigen via hIg.

Example 1 demonstrated an increased immune responses to hIg after DNA and protein immunisation was obtained with targeting ligand-hIg fusions. The following Examples were conducted to determine if antigens other that hIg could be used for increased immune responses. These data were obtained by the addition of antigens to the C-terminus of hIg which could facilitate dimer formation as was found with hIg alone. A gly-gly-gly-gly-thr spacer was introduced between hIg and the antigens. Whilst these constructs have been used it will be appreciated that responses may be improved by routine optimisation. This optimisation may involve modification of the constructs as envisaged within the present invention eg different targeting molecules, different sequences which facilitate multimerisation, different linkers etc.

EXAMPLE 3

Use of CTLA4 to accelerate immune responses against the host protective antigen of *Taenia ovis* known as 45W

Introduction

The 45W antigen is a putative membrane glycoprotein present in, or underlying the tegument of, the *Taenia ovis* oncosphere. *T. ovis* is a pathogen of sheep which causes commercial losses of mutton and wool in New Zealand and other important sheep growing countries. Early immunisation studies using 45W protein partially purified from *T. ovis* revealed that it was a promising vaccine antigen. Subsequent field trials using recombinant forms of 45W, expressed in Escherichia coli, as a vaccine reported very high levels (about 95%) of protection[13]. The 45W antigen was used as a DNA vaccine in sheep and low levels of antibody, measured using a recombinant form of 45W, was observed[14].

Materials and Methods

Plasmids containing the CMV promoter and the genes encoding CTLA4, the Fc portion of human immunoglobulin (hIg) and the CD5 signal peptide were described above. The gene encoding 45W was obtained from Dr. Marshall Lightowlers (Dept. Veterinary Science, University of Melbourne).

Inbred Balb/c mice of 6-8 weeks of age were obtained from the Dept. Microbiology and Immunology Animal House, University of Melbourne.

Standard DNA manipulation and CsCl purification techniques were used. The gene encoding 45W was ligated into two DNA vaccines. Construct pCI::mCTLA4-hIg-45W expressed a fusion protein which comprised the CTLA4 signal peptide, mouse CTLA4 ectodomain, hIg and the 45W antigen. Construct pCI::CD5L-hIg-45W expressed a fusion protein which contained the signal peptide from CD5, hIg and the 45W antigen.

DNA (100μg) was injected into the quadriceps of mice on days 0 and day 28. Sera was obtained at appropriate intervals post vaccination and analysed for total antibodies specific for recombinant 45W antigen in a

titration ELISA[15] using horseradish peroxidase conjugated anti-mouse immunoglobulins.

Purified recombinant 45W(His)$_6$ was obtained from *E. coli* according to Rothel *et al.*[14] using a polyhistidine 'tag' and nickel affinity chromatography.

Results

Mice were immunised with the DNA vaccines pCI::mCTLA4-hIg-45W, or pCI::CD5L-hIg-45W (Figure 10). Mice received 100ug of DNA on days 0 and 28 and were bled at weekly intervals. Mice receiving the DNA vaccine which expressed CTLA4 fused to the hIg/45W vaccine developed a more rapid antibody response than the mice which received a similar plasmid vaccine construct ie. pCI::CD5L-hIg-45W which did not contain the CTLA4 gene. The mice receiving the vaccine with CTLA4 produced serum antibodies of high titre (ie.≥10,000) on days 7, 14 and 28. In comparison, mice which received the construct lacking CTLA4 did not produce high titre antibodies (ie. titre ≥10,000) until after the second immunisation on day 28. All mice(ie. 5/5) which received the DNA vaccine containing CTLA4 produced 45W-specific antibodies by 7 days post immunisation whereas only 1/5 animals which received the equivalent DNA vaccine lacking CTLA4 produced antibodies at day 7 post immunisation. The data was analysed using Student's t-test.

A second trial was undertaken where mice received either 20μg of purified recombinant 45W(His)$_6$ protein in Complete Freund's Adjuvant, or the CTLA4 DNA vaccine (ie. pCI::mCTLA4-hIg-45)(Figure 11). The serum antibody response was examined on days 0, 2, 5, 8, 14 and 28. The serum antibody response specific for 45W was higher at day 8 in DNA vaccinated mice than in mice which received the 45W protein vaccine.

Discussion

The murine serum antibody response to 45W DNA vaccination was accelerated by fusion of CTLA4 to the hIg-45W fusion protein. The antibody response to 45W correlates with protection in sheep against *T. ovis* disease. Addition of CTLA4 led to a more rapid high titre response, with a shorter

unprotected period following immunisation. The effect of CTLA4 on the magnitude of the anti-45W response was not as dramatic as the effect on human Ig described above. This may have been due to the conformational restraints from the fusion of the various molecules or some inherent property of the 45W antigen. Furthermore, the immunisation protocol employed in this Example differed from Example 1 in that boosting occurred at 4 rather than two weeks. Due to the more rapid kinetics of the response via CTLA4 targeting boosting may not have been optimum and thus the magnitude was not effected.

Example 4

Use of CTLA4 with AMA1 to protect against *Plasmodium chabaudi adami* in mice

Introduction

AMA1[16] is a candidate vaccine antigen against malaria. We have evidence that domain3 of AMA1 folds independently and as such may be a good candidate in producing a fusion protein with hIg. However, although AMA1 has been shown to confer protection in mouse malaria[17], we are unaware of any work that has shown domain3 to be protective.

Materials and Methods

Plasmids containing the CMV promoter and the genes encoding CTLA4, the Fc portion of human immunoglobulin (hIg) and the CD5 signal peptide were described above. Domain 3 of AMA-1 from the *Plasmodium chabaudi adami* DS strain[16] was fused to CTLA4Ig and CD5LIg (pCI::mCTLA4-hIg-AMA and pCI::CD5L-hIg-AMA). The plasmid pCI::mCTLA4-hIg was used as a negative control.

Inbred female Balb/c mice of 6-8 weeks of age were used.

DNA (100ug) was injected into the quadriceps of mice on day 0 only. Mice were challenged with *Plasmodium chabaudi adami* DS and the number of deaths recorded.

Antibody titres were measured by ELISA using refolded *E.coli* expressed entire ectodomain of AMA1[17]. The titres were expressed as the log of the reciprocal of the last serum dilution to give an OD >0.1.

Results

In the first trial, there were 8 mice per group. A single immunisation with the DNA vaccine pCI::mCTLA4-hIg-AMA afforded partial protection against an intraperitoneal challenge of 100,000 parasites (Figure 12). Challenge was performed 14 days after immunisation. This was significant (log rank test; $p<0.05$) from the control pCI::mCTLA4-hIg group. There was a clear indication that the CTLA4 conferred better protection than the pCI::CD5L-hIg-AMA group, although this did not reach statistical significance. The antibody titres (Figure 13) show that the CTLA4 targeting ligand enhances the antibody response to AMA1 ($p<0.005$).

A second trial was undertaken with larger group (16/group) and with an intravenous challenge of 10,000 parasites. No protection was seen in this second trial.

Discussion

In the first trial, CTLA4 conferred some protection against malaria by domain3 of antigen AMA1. This was not found in the second trial. We do not know why there was a difference between the two trials. Because there is not a full set of antibody data for comparison, we do not know whether the level of antibody achieved was sufficient in the mice that died in trial two, or whether the effect was due the different route of challenge. We also do not know how effective CTLA4IgAMA may be when booster doses are given.

Example 5
Use of CTLA4 in influenza infection in mice

Introduction

As an additional model for testing protective efficacy we have used influenza infection of the murine respiratory tract. The influenza

haemagglutinin (HA) gene was cloned behind targeting molecules and the resulting DNA vaccines examined for their ability to generate higher anti-viral antibody titres and afford greater protection against live viral challenge compared to control vaccines not expressing the targeting molecule.

Materials and Methods

Virus

The type A influenza virus used in this study was PR8 = A/Puerto Rico/8/34 (H1N1). Virus was grown in the allantoic cavity of 10-day embryonated hens' eggs for 2 days at 35°C. The allantoic fluid was collected and clarified by centrifugation (2000g, 15 mins, 4°C). Aliquots of allantoic fluid containing infectious virus were stored at -70°C and used for immunisation and challenge of mice. Purified PR8 virus used in ELISA assays was obtained as zonally purified stocks from CSL Ltd, Parkville, Victoria, Australia. The haemagglutination assay[18] was used to quantitate virus and titres are expressed in haemagglutinating units (HAU) per ml.

Immunisation

Groups of 10 BALB/c mice were immunised intramuscularly (i.m.) under anaesthesia on day 0 with 0.1 ml of DNA vaccine containing 50μg of plasmid. The constructs used for immunisation were pCI::CD5L-hIg-HA, pCI::mCTLA4-hIg-HA, which were based on PR8 HA lacking the signal and transmembrane sequences, and pCI::CD5L-hIg-SIINFEKL (expressing an 8 amino acid sequence from chicken ovalbumin) as a negative control. As a positive control, a group of 10 BALB/c mice were infected intranasally (i.n.) with 50 plaque forming units (pfu) of infectious PR8 virus and another group immunised subcutaneously with 1μg of β-propiolactone (BPL)-inactivated and sodium taurodeoxycholate-disrupted PR8 virus (split virus). Serum samples were collected from all mice on days 7, 14 and 54 and mice were then challenged on day 65.

Intranasal challenge of mice and preparation of mouse lung extracts

Penthrane anaesthetised mice were challenged with 50 pfu of infectious PR8 influenza virus i.n.. Each mouse received 50 μl of virus in the

form of allantoic fluid diluted in phosphate buffered saline (PBS). Five days after challenge, mice were killed by cervical dislocation and lungs were removed and transferred aseptically to bottles containing 1.5 ml Hanks Balanced Salt Solution (HBSS), supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml and 30 μg of gentamicin per ml. Lung homogenates were prepared using a tissue homogeniser. Each lung suspension was then centrifuged at 300 x g for 5 minutes and the supernatants were removed, aliquoted and stored at -70°C prior to assay for infectious virus.

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) was performed as previously described by Jackson *et al.*[19] using 96-well polyvinyl microtitre trays (Dynatech, Australia) coated with a solution containing 50 HAU of purified PR8 virus per well. Antibody titres are expressed as the reciprocal of the antibody dilution giving an absorbance of 0.2 units.

Plaque assay for infectious virus

Virus titres were determined by plaque assay on monolayers of Madin-Darby canine kidney (MDCK) cells in 35 mm petri dishes (Nunc, Roskilde, Denmark). The culture medium was RPMI-1640 supplemented with 2 mM glutamine, 2 mM sodium pyruvate, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 30 μg of gentamicin per ml and 10% (vol/vol) foetal calf serum (heat inactivated at 56°C for 30 min). Monolayers were washed with serum-free RPMI-1640 containing antibiotics and inoculated in duplicate with 100 μl of dilutions of lung homogenates in the same medium. After allowing 45 minutes in a humidified incubator (37°C, 5% CO_2) for virus adsorption, 3 ml of agarose overlay medium at 45°C was added. Incubation was continued for a further 3 days and plaques counted. The agarose overlay medium was Leibovitz L-15 medium pH 6.8 (Gibco Laboratories, U.S.A.) supplemented with 100 U penicillin per ml, 100 μg of streptomycin per ml, 0.01 M HEPES buffer pH 6.8 (Calbiochem, Australia), 0.1% trypsin-TPCK (Worthington, Biological Systems Inc., U.S.A.) and 0.9% agarose (ICN Biomedicals Sydney, Australia).

Statistical analysis

The data were analysed using the nonparametric Mann-Whitney U test which compares two sets of unpaired samples. The null hypothesis is that the two population medians are equal and the resultant P value for particular comparisons is given.

Results and Discussion

Serum antibody responses of mice

Sera collected from mice immunised with the DNA constructs, split PR8 virus in PBS, or infectious PR8 virus were assayed for anti-viral antibody by ELISA. On day 7 after priming, only mice immunised with infectious PR8 virus exhibited anti-viral antibody titres significantly higher than the background titres detected in mice given the control DNA construct pCI::CD5L-hIg-SIINFEKL ($p=0.0002$). However by day 14, in addition to the high antiviral antibody titres detected in the virus infected mice, antibody titres of mice immunised with the pCI::mCTLA4-hIg-HA construct were significantly higher than those of mice given the control DNA constructs pCI::CD5L-hIg-HA ($p=0.0003$) or pCI::CD5L-hIg-SIINFEKL ($p=0.0004$) (Fig. 14). Furthermore, mice immunised with the pCI::mCTLA4-hIg-HA construct had comparable levels of antiviral antibody to mice given the split virus vaccine ($p=0.97$). The antiviral antibody titres of sera collected on day 54 post-priming were also determined (Fig 15). Overall, the day 54 titres were similar to those measured in sera collected on day 14, and the level of antibody in the day 54 sera of mice given the pCI::mCTLA4-hIg-HA construct remained significantly higher than that of mice given pCI::CD5L-hIg-HA ($p=0.009$) or pCI::CD5L-hIg-SIINFEKL ($p=0.0028$), and comparable to split virus ($p=0.85$).

Ability of PR8 HA constructs to elicit protective immunity

Protection of vaccinated mice from influenza infection was assessed by examining the ability of mice to clear a challenge dose of virus from their lungs by five days post-infection. It should be noted that both antibody and cytotoxic T cell-mediated responses can lead to viral titre reduction within this 5 day interval. Mice were challenged on day 65 post-priming and the titre of virus in their lung was determined by a plaque assay. Figure 16

shows that all mice immunised with infectious virus were able to clear the challenge dose of virus. Of the mice given DNA constructs, the lung virus titres of mice immunised with the pCI::mCTLA4-hIg-HA construct were significantly lower than those of mice immunised with either pCI::CD5L-hIg-HA ($p=0.0004$) or pCI::CD5L-hIg-SIINFEKL ($p=0.0002$). Also the level of clearance observed in the pCI::mCTLA4-hIg-HA construct-immunised mice was almost as good as that seen in mice given the split virus vaccine.

Conclusions

pCI::CTLA4-hIg-HA conferred higher antibody levels and better protection against challenge compared with the control vector pCI::CD5L-hIg-HA demonstrating the immune enhancing effect of the incorporation of the targeting molecule.

Example 5

Use of CTLA4 in *Corynebacterium pseudotuberculosis* in sheep.

Introduction

Corynebacterium pseudotuberculosis is the causative agent of caseous lymphadenitis (CLA) in sheep. Established infection by these bacteria leads to the formation of abscesses in the lymph nodes, especially the draining lymph node of the site of infection. Phospholipase D (PLD) has been characterised as a virulence factor and a protective antigen for CLA. Indeed formalin-treated PLD[20] or genetically toxoided PLD (ΔPLD[22]) has been shown to protect sheep from CLA.

We have used the genetically toxoided PLD as the basis for our DNA vaccination approach and investigated whether the addition of bovine (b)CTLA4-hIg or hIg alone to the ΔPLD construct enhanced the immune response to PLD and to hIg.

Materials and methods.

DNA constructs

Using the known sequence of bCTLA4 (GenBank accession number X15070), the bCTLA4 gene was isolated from bovine peripheral blood mononuclear cells. A PCR product of bCTLA-4 (729 bp) was cloned into the Zeroblunt TM cloning vector according to the manufacturer's instructions (Invitrogen) and sequenced using the Applied Biosystems automated sequencer. The sequence of bCTLA4 was found to be identical to the published sequence.

The following constructs were generated in the pCI vector for DNA immunisation:

pCI::bCTLA4-hIg-ΔPLD
pCI::ΔPLD
pCI::bCTLA4-hIg
pCI::CD5L-hIg-ΔPLD*

* CD5L refers to the leader sequence of the CD5 molecule allowing the huIg-ΔPLD protein to be secreted.

Experimental animals and immunisation regimen

Cross-bred ewes aged 12 weeks were used in the challenge trial. 10 animals were allocated randomly to each group. Animals were pre-screened for the presence of antibodies to PLD and to *Corynebacterium pseudotuberculosis* lysate. Positive animals were excluded from the trial. Shearing, vaccination and tail docking of animals was avoided to minimise risk of infection with *Corynebacterium pseudotuberculosis.*

Animals were injected intra-muscularly with 500 μg LPS free pCI plasmid DNA (coding for either pCI::bCTLA4-hIg-ΔPLD, pCI::ΔPLD, pCI::bCTLA4-huIg or pCI::CD5L-huIg-ΔPLD) in 5 ml of PBS. Control animals received either Glanvac or were left un-immunised. All animals received the same vaccine, at the same dose, 4 weeks later.

Challenge

Bacterial cultures of wild type *Corynebacterium pseudotuberculosis* were grown at 37° C in Brain heart infusion broth (Difco Laboratories) containing 0.1% Tween 80 (BHI).

All sheep were challenged 6 weeks after primary immunisation using a 1 ml dose of 10^6 CFU of *Corynebacterium pseudotuberculosis* injected just above the coronet of the right hind lateral claw.

Immunological assays

Sera were collected from the sheep at weekly intervals and assayed for the presence of antibodies to genetically detoxified PLD (ΔPLD) and hIg using an ELISA. Plates were coated with 1/50 of culture supernatant from ΔPLD expressing *Corynebacterium pseudotuberculosis* or 5 g/ml hIg protein. The sera were diluted in two fold steps starting at 1/100 and 1/10 for the detection of anti-ΔPLD and anti-hIg antibodies respectively. Titres were calculated by linear regression on a double logarithmic scale in the linear part of the graph. The titre was defined as the dilution, which resulted in an O.D. 0.3 in the ELISA.

T cell proliferation assays were performed in triplicates using 2 concentrations of ΔPLD (1/50 and 1/250) or hIg (5 g/ml and 25 g/ml) as an antigen. PBMC were purified by ficoll gradient and cultured *in vitro* for 3 days. The cultures were pulsed with 3H-methyl-thymidine for 18 hours before being harvested on glass fibre filters and radioactive incorporation assessed. The results are presented as stimulation indices (i.e. ratio between counts obtained with antigen over counts obtained without antigen).

Statistical analysis was performed using the Systat program. The non-parametric Mann-Whitney U test was used to calculate significance. p values below 0.05 were considered significant.

Results

Anti-hIg antibody levels

The antibody titres to human immunoglobulin (hIg) reflect the immune response to the DNA vaccination against the hIg part of the fusion protein in the case of bCTLA4-hIg-ΔPLD, CD5L-hIg-ΔPLD and bCTLA4-hIg. By comparing the response to hIg from the animals injected with pCI::bCTLA4-hIg-ΔPLD to these injected with pCI::CD5L-hIg-ΔPLD it is possible to specifically evaluate the effect of bCTLA4 targeting on the immunogenicity of hIg. Results shown in Fig 17 indicate that the antibody

response to hIg in animals injected with pCI::bCTLA4-hIg-ΔPLD (filled squares) is both earlier and stronger than the anti-hIg response induced in animals immunised with pCI::CD5L-hIg-ΔPLD (closed circles). The Mann-Whitney U test indicates a statistically significant difference for weeks 3 and 4. Corroborating these results the anti-hIg response in the group injected with pCI::bCTLA4-hIg (closed triangles) is also earlier and stronger than the response in animals injected with pCI::CD5L-hIg-ΔPLD.

Anti-ΔPLD antibody levels

Immunisation with the detoxified-PLD protein antigen (Glan-Vac) resulted in little or no detectable antibodies during the first 7 weeks after immunisation. Two weeks after challenge antibody levels increased dramatically. This is consistent with previously reported results[21]

All groups immunised with pCI encoding the ΔPLD antigen either alone or as a fusion protein with bCTLA4-hIg or with CD5L-hIg, resulted in similar kinetics of antibody production. Indeed, no significant anti-ΔPLD antibody levels were detected until 2 weeks post-challenge (i.e. week 8). At each time point there was no significant difference between the level of antibodies induced by the different pCI constructs, indicating that all constructs have a similar ability to induce immune memory to ΔPLD. However, this result is not surprising in light of the fact that detoxified-PLD is not a classical antigen in that two doses of the protein antigen (Glan-Vac group) failed to induce substantial antibody levels. This result has also been reported by others[22]. Thus detoxified-PLD seems to result in the induction of immune memory without induction of high antibody titres. This immune memory is then activated during the early stage of the challenge by PLD expressed by *Corynebacterium pseudotuberculosis*. It is interesting to note that ΔPLD expressed in eukaryotic cells (COSm6 cells) has a slightly larger size compared to ΔPLD expressed in *Corynebacterium pseudotuberculosis* (Fig 18). This difference can be accounted for by the possible glycosylation of ΔPLD in eukaryotic cells. As in the case with anti-hIg antibody levels it is noted that sheep, in contrast to mice, do not produce antibodies over prolonged periods of time following DNA vaccination.

T cell responses to ΔPLD and hIg.

T cell responses to ΔPLD and hIg were analysed at 3 weeks, 6 weeks and 9 weeks. No significant antigen specific proliferation, to these two antigens could be demonstrated in peripheral blood mononuclear cells (PBMC) at any time point. This is most likely due to technical difficulties since the kinetics of the anti ΔPLD response indicate the likely presence of memory T cells in the vaccinated animals.

Anti-ΔPLD response subsequent to challenge with virulent *C. pseudotuberculosis.*

Challenge was performed at 6 weeks post-immunisation (i.e. two weeks post-booster immunisation). Observation of the site of inoculation at week 10 did not reveal any correlation with immunisation regime. Except in the case of the unvaccinated control and pCI::bCTLA4-hIg immunised animals, the antibody response to ΔPLD increased two weeks post-challenge (week 8). This indicates that the immune memory induced by the immunisation with DNA can be boosted with ΔPLD produced by *Corynebacterium pseudotuberculosis.* This indicates that although ΔPLD produced by DNA vaccination is most likely glycosylated there is still cross-reaction with bacterial wild-type PLD.

In the un-immunised control animals the level of anti-ΔPLD antibodies remains low until 3 to 4 weeks post-challenge (week 9). This kinetics of antibody appearance has been described previously[22].
Interestingly the antibody levels in the animals primed with ΔPLD by DNA vaccination or detoxified-PLD by Glan-Vac injection, diminished after week 8 suggesting that the PLD antigen is no longer boosting the immune response. This may be due to a diminished level of PLD secretion due to the animals clearing *Corynebacterium pseudotuberculosis.*

Protection from challenge with virulent *Corynebacterium pseudotuberculosis.*

At week 12 post vaccination (6 weeks post challenge) necropsy of all animals was performed to evaluate the protective efficacy of the immunisation protocol. Both left and right popliteal, inguinal and prefemoral lymph nodes were dissected and visually assessed for the characteristic abscesses induced by *Corynebacterium pseudotuberculosis.* The lungs were palpated to detect abscesses and no lung abscesses were

found in any of the animals. Protection was defined as the absence of the characteristic *Corynebacterium pseudotuberculosis* abscesses in any of the lymph nodes. In two sheep small dry lesions were observed in the draining popliteal lymph node, clearly distinct from the abscesses in the other animals. These lesions were most likely foci of *Corynebacterium pseudotuberculosis* which were regressing in the face of an effective immune response and these animals were also scored as "protected". From Fig 19 it can be seen that while about 10 % of the unvaccinated animals did not develop lesions, 90 % were protected by vaccination with Glan-Vac. Only 1 animal out of 10 injected with pCI::CTLA4-hIg did not have abscesses in the lymph nodes. All animals vaccinated with DNA encoding for ΔPLD (pCI::bCTLA4-hIg-ΔPLD, pCI::ΔPLD and pCI::CD5L-hIg-ΔPLD) were afforded some protection. The level of protection ranged from 40 to 70% with the highest degree of protection observed in the group injected with pCI::CTLA4-hIg-ΔPLD.

Conclusion

This study has examined the ability of bCTLA4 to increase the immune response to antigens during DNA immunisation in sheep. bCTLA4 has the ability to accelerate and increase the immune response to hIg. Challenge with *Corynebacterium pseudotuberculosis* indicated that DNA vaccination could induce a protective immune response. When comparing the protection obtained with pCI::bCTLA4-hIg-ΔPLD and pCI::CD5L-hIg-ΔPLD a substantial difference was observed.

The protection induced by CD5L-hIg-ΔPLD is lower than the protection induced by ΔPLD on its own. This difference may be due to the fact that the ΔPLD molecule is much smaller than the bCD5L-hIg-ΔPLD and hence could be expressed in an acceptable conformation more easily. One could therefore expect that the bCTLA4-hIg-ΔPLD molecule which is even larger would be even more difficult to express. Hence the fact that the bCTLA4-hIg-ΔPLD molecule induced higher levels of protection indicates that the CTLA4 molecule effectively increases the immunogenicity of the fusion protein. It can be reasonably expected that by improving the level of expression and folding of the molecule even better protection could be

obtained. One way of achieving this would be to reduce the size of the hIg portion of the fusion protein.

Example 7

Vaccination of C3H/He mice with DNA encoding the Parasite Surface Antigen 2 (PSA2) of *Leishmania major*.

Introduction

Protection against infection with this obligatory intracellular parasite is provided by CD4+ T cells of the macrophage activating, Th1 type. We have shown that injection of plasmid DNA encoding full length PSA2 induced significant protection against a challenge infection in C3H/He mice. This protection correlated with the induction of a very low, but consistent Th1 type of immune response, ie induction of T cells secreting interferon gamma, but no IL-4. Here we aim to examine the ability of CTLA4-Ig to improve the level of protection.

Materials and methods

PSA2 encoding residues 33-357 (missing the leader and gpi signal) was fused C-terminal to either pCI::CD5L-hIg or pCI::mCTLA4-hIg. DNA encoding a secreted form of PSA2 (residues 1-357) was also made (pCI::PSA2)

Groups of 8 mice were injected with 100 µg DNA in 100 µl phosphate buffered saline (PBS) intramuscularly twice at two week intervals. Two weeks after each injection the mice were bled and the serum tested for antibodies to PSA-2. Two weeks after the second injection the mice were infected intradermally with 100,000 promastigotes. The development of lesions at the site of infection was monitored weekly and scored according to size. Parasite burdens in the lymph nodes draining the lesion were determined by limiting dilution analysis at 7 weeks after challenge infection.

Results

Antibody production

Antibodies were measured only by ELISA OD at a single point. Two weeks after the first immunisation, 4 of 8 mice immunised with pCI::mCTLA4-hIg-PSA2 and 3 of 8 mice given pCI::CD5L-hIg-PSA2 produced significant antibody at a dilution of 1:500. However, after the second injection of DNA mice immunised with pCI::mCTLA4-hIg-PSA2, pCI::CD5L-hIg-PSA2 and our own secreted form of PSA-2 showed significant levels of antibody at this dilution. The PBS control had background antibody.

Protection from infection

Mice immunised with DNA encoding pCI::CD5L-hIg-PSA2 and the controls PBS and vector DNA developed lesions at the site of infection 1 week after challenge. Mice immunised with pCI::mCTLA4-hIg-PSA2 or pCI::PSA2 developed lesions only 3 weeks after infection and the size of the lesions was smaller compared to the rest. Mice immunised with pCI::mCTLA4-hIg-PSA2 or pCI::PSA2 also had the smallest number of mice which developed lesions with only 5 of 8 mice showing any lesions at the peak of the disease curve (Fig 20). Notably, pCI::mCTLA4-hIg-PSA2 conferred better protection than pCI::CD5L-hIg-PSA2 ($p=.0001$; log rank test).

Summary

The ability to overcome the problem of low or absent responsiveness in DNA immunisation by antigen targeting enhances the potential of genetic vaccines. The present inventors also show that intramuscular injection of DNA can also be employed to deviate immune responses to the same antigen allowing for the development of vaccines in which the response most likely to confer protection can be generated.

Intramuscular injection of expression plasmids shows great potential for genetic vaccination. The present inventors have shown that fusion proteins consisting of antigen and cell surface receptor ligands could deliver antigen to sites of immune induction which enhance the immune response and possibly the efficacy of genetic vaccines. As set out above mice were

immunized with plasmids encoding Fc fragment of human IgG1 as antigen. This Ig fragment was fused with CTLA4 (CTLA4Ig) for delivery to antigen presenting cells (APC) expressing B-7, or with L-selectin (L-SELIg) for delivery to high endothelial venule cells of lymph nodes. L-selectin binds CD34 and MadCAM-1 and so could target any lymphoid organ with these receptors (12). Enhanced antibody responses were shown in both the CTLA4Ig and L-SELIg immunized mice, 1000 and 100 fold respectively at 4 weeks. Moreover the response after CTLA4Ig immunisation was the most rapid. Immune deviation from an IgG2a to an IgG1 dominated response occurred in CTLA4Ig immunized mice and allows for the development of genetic vaccines in which the response most likely to confer protection can be generated.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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(ii) TITLE OF INVENTION: Enhancement of Immune Response using Targeting Molecules

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: AU PO 5891
(B) FILING DATE: 27-MAR-1997

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: AU PP 1830
(B) FILING DATE: 13-FEB-1998

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11265 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CLAIMS:

1. A DNA molecule for use in raising an immune response to an antigen, the DNA molecule including a first sequence encoding a targeting molecule, a second sequence encoding the antigen or an epitope thereof, and optionally a third sequence encoding a polypeptide which promotes dimerisation or multimerisation of the product encoded by the DNA molecule.

2. A DNA molecule as claimed in claim 1 in which the antigen or epitope thereof encoded by the second sequence is a polypeptide which promotes dimerisation or multimerisation of the product encoded by the DNA molecule and the third sequence is absent.

3. A DNA molecule as claimed in claim 1 in which the third sequence is present.

4. A DNA molecule as claimed in any one of claims 1 to 3 in which the targeting molecule encoded by the first sequence is a ligand which targets lymphoid cells, lymphoid sites or antigen presenting cells.

5. A DNA molecule as claimed in any one of claims 1 to 4 in which the targeting molecule encoded by the first sequence is selected from the group consisting of CTLA4, L-selectin, CD40L, OX40, CD28, and antibodies to receptors on antigen presenting cells.

6. A DNA molecule as claimed in any one of claims 1 to 5 in which the targeting molecule encoded by the first sequence is CTLA4.

7. A polypeptide, the polypeptide being encoded by the DNA molecule as claimed in any one of claims 1 to 6.

8. A vector including the DNA molecule as claimed in any one of claims 1 to 6.

9. A composition for use in raising an immune response in an animal the composition including the DNA molecule as claimed in any one of claims 1 to 6 or the vector as claimed in claim 8 and an acceptable diluent or excipient.

10. A composition for use in raising an immune response in an animal the composition including the polypeptide as claimed in claim 7 and an acceptable diluent or excipient.

11. A method of raising an immune response in an animal, the method comprising administering to the animal the composition as claimed in claim 9 or claim 10.

12. A method of deviating the immune response to an antigen in an individual, the method comprising administering to the individual a DNA molecule including a first sequence encoding CTLA4, a second sequence encoding the antigen or an epitope thereof, and optionally a third sequence encoding a polypeptide which promotes dimerisation or multimerisation of the product encoded by the DNA molecule.

13. A method as claimed in claim 12 in which the antigen or epitope thereof encoded by the second sequence is a polypeptide which promotes dimerisation or multimerisation of the product encoded by the DNA molecule and the third sequence is absent.

14. A method as claimed in claim 12 in which the third sequence is present.

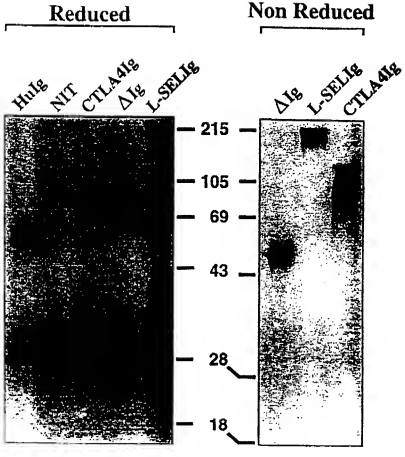
Reduced
Non Reduced
HuIg
NIT
CTLA4Ig
ΔIg
L-SELIg
ΔIg
L-SELIg
CTLA4Ig
215
105
69
43
28
18

FIGURE 1

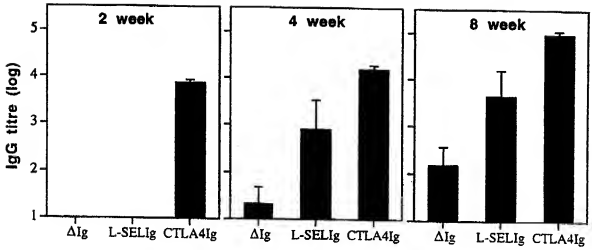
2 week
4 week
8 week
IgG titre (log)
5
4
3
2
1
ΔIg
L-SELIg
CTLA4Ig
ΔIg
L-SELIg
CTLA4Ig
ΔIg
L-SELIg
CTLA4Ig

FIGURE 2

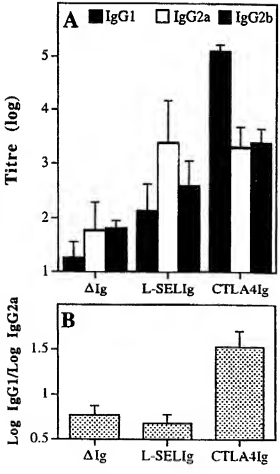
A
IgG1
IgG2a
IgG2b
Titre (log)
5
4
3
2
1
ΔIg
L-SELIg
CTLA4Ig
B
Log IgG1/Log IgG2a
1.5
1
0.5
ΔIg
L-SELIg
CTLA4Ig

FIGURE 3

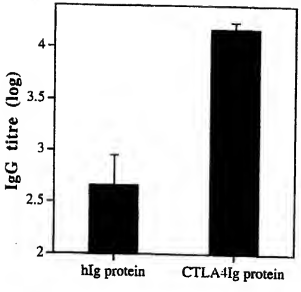

FIGURE 4

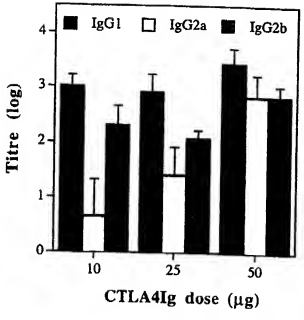

FIGURE 5

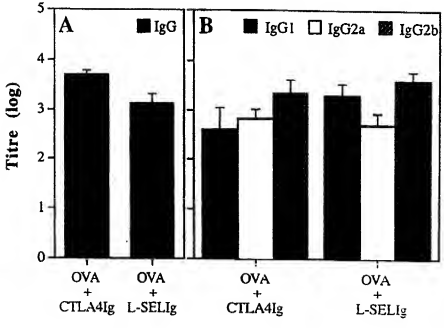
A
IgG
B
IgG1
IgG2a
IgG2b
Titre (log)
5
4
3
2
1
0
OVA + CTLA4Ig
OVA + L-SELIg
OVA + CTLA4Ig
OVA + L-SELIg

FIGURE 6

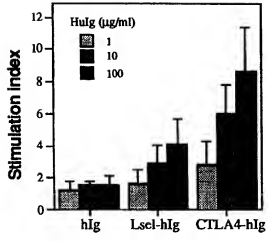
Stimulation Index
12
10
8
6
4
2
0
HuIg (µg/ml)
1
10
100
hIg
Lsel-hIg
CTLA4-hIg

FIGURE 7

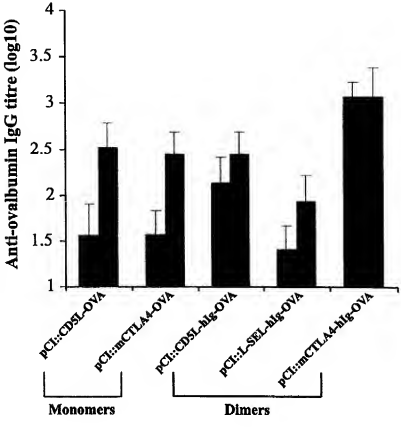
Anti-ovalbumin IgG titre (log10)
4
3.5
3
2.5
2
1.5
1
pCI::CD5L-OVA
pCI::mCTLA4-OVA
pCI::CD5L-hIg-OVA
pCI::L-SEL-hIg-OVA
pCI::mCTLA4-hIg-OVA
Monomers
Dimers

FIGURE 8

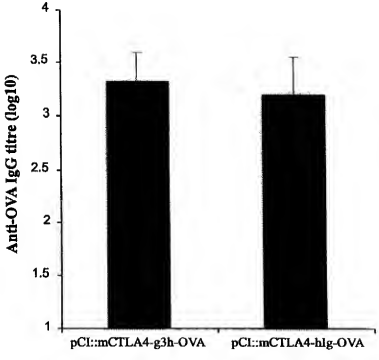
Anti-OVA IgG titre (log10)
4
3.5
3
2.5
2
1.5
1
pCI::mCTLA4-g3h-OVA
pCI::mCTLA4-hIg-OVA

FIGURE 9

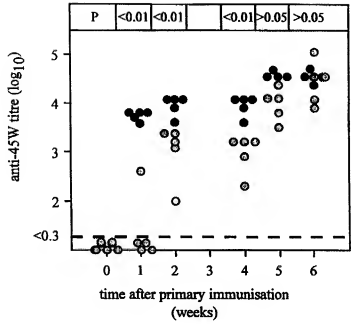
P
<0.01
<0.01
<0.01
>0.05
>0.05
anti-45W titre (log10)
5
4
3
2
<0.3
0
1
2
3
4
5
6
time after primary immunisation
(weeks)

FIGURE 10

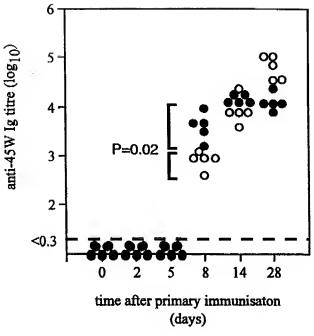
6
5
4
3
2
<0.3
anti-45W Ig titre (log_{10})
P=0.02
0
2
5
8
14
28
time after primary immunisaton
(days)

FIGURE 11

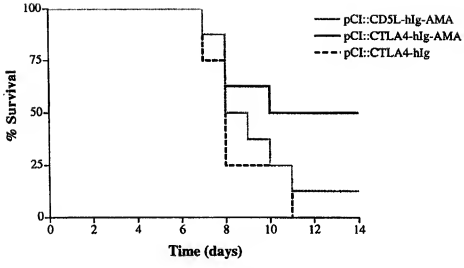
100
75
50
25
0
% Survival
0
2
4
6
8
10
12
14
Time (days)
pCI::CD5L-hIg-AMA
pCI::CTLA4-hIg-AMA
pCI::CTLA4-hIg

FIGURE 12

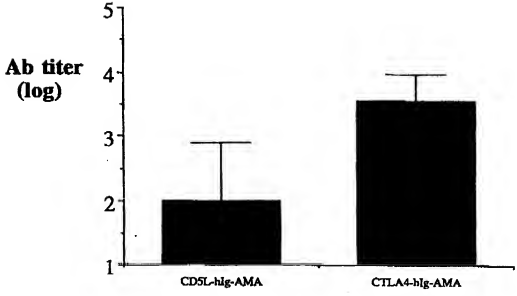

FIGURE 13

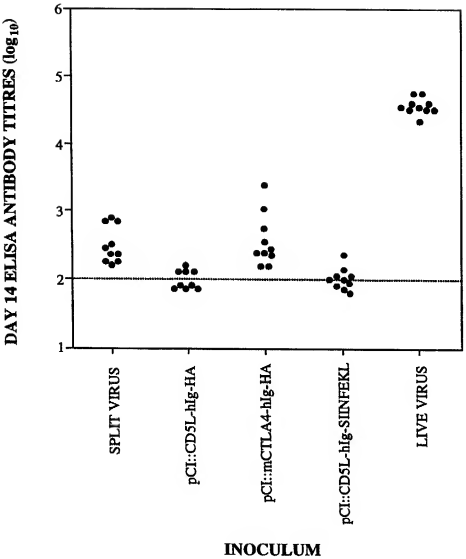
DAY 14 ELISA ANTIBODY TITRES (log_{10})
6
5
4
3
2
1
SPLIT VIRUS
pCI::CD5L-hIg-HA
pCI::mCTLA4-hIg-HA
pCI::CD5L-hIg-SIINFEKL
LIVE VIRUS
INOCULUM

FIGURE 14

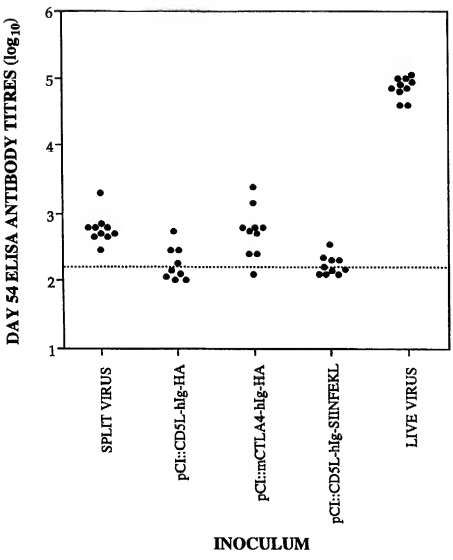
DAY 54 ELISA ANTIBODY TITRES (log_{10})
6
5
4
3
2
1
SPLIT VIRUS
pCI::CD5L-hIg-HA
pCI::mCTLA4-hIg-HA
pCI::CD5L-hIg-SIINFEKL
LIVE VIRUS
INOCULUM

FIGURE 15

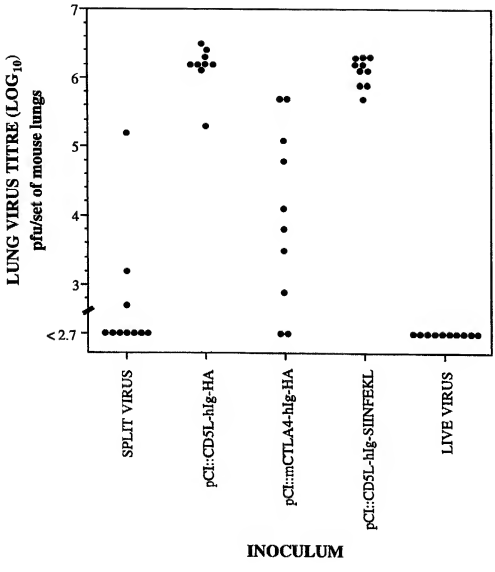
LUNG VIRUS TITRE (LOG_{10})
pfu/set of mouse lungs
7
6
5
4
3
< 2.7
SPLIT VIRUS
pCI::CD5L-hIg-HA
pCI::mCTLA4-hIg-HA
pCI::CD5L-hIg-SIINFEKL
LIVE VIRUS
INOCULUM

FIGURE 16

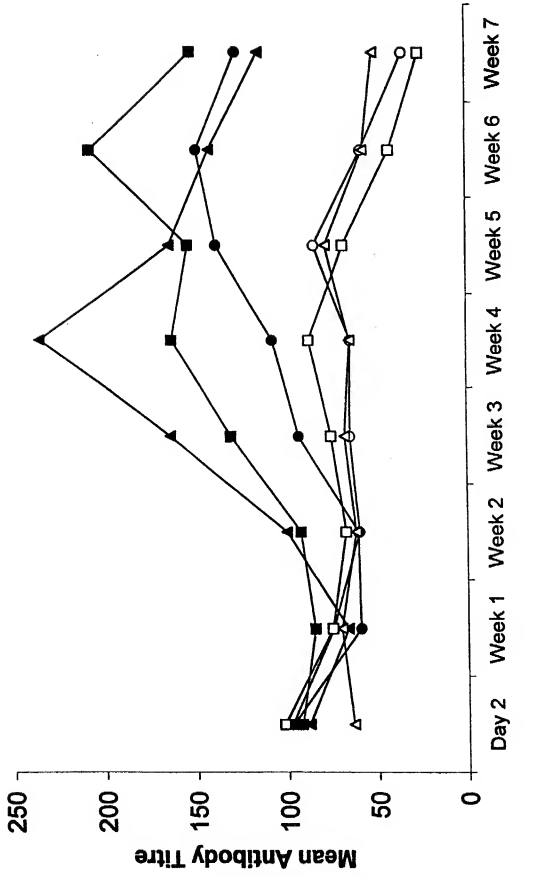
Mean Antibody Titre
250
200
150
100
50
0
Day 2
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
Week 7

FIGURE 17

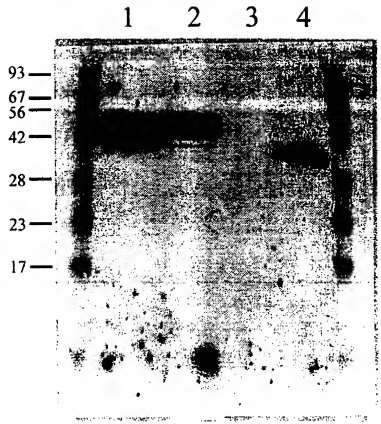

FIGURE 18

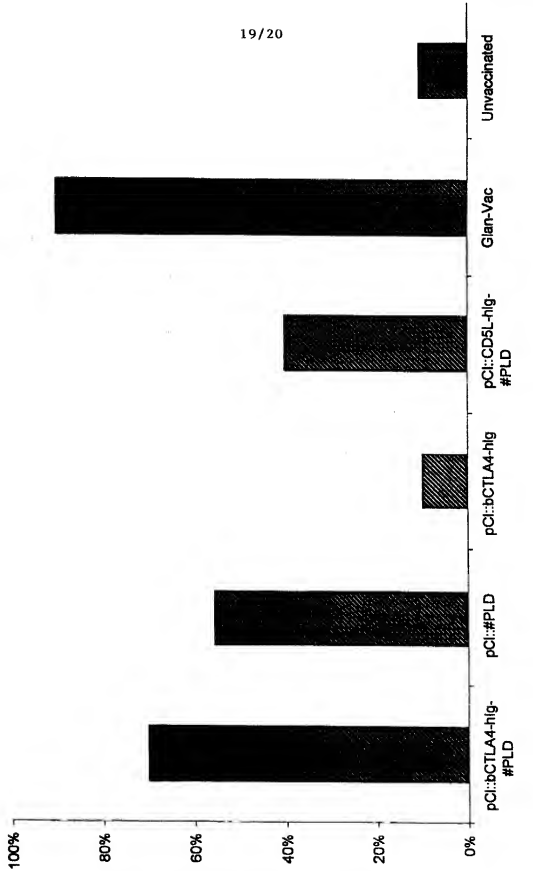
Percentage protected animals
100%
80%
60%
40%
20%
0%
pCI::bCTLA4-hIg-#PLD
pCI::#PLD
pCI::bCTLA4-hIg
pCI::CD5L-hIg-#PLD
Glan-Vac
Unvaccinated

FIGURE 19

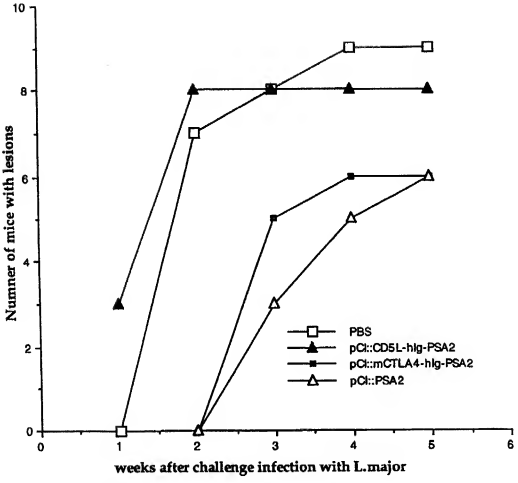
Numner of mice with lesions
weeks after challenge infection with L.major
PBS
pCI::CD5L-hIg-PSA2
pCI::mCTLA4-hIg-PSA2
pCI::PSA2
0
2
4
6
8
10
0
1
2
3
4
5
6

FIGURE 20

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00208

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/62; C07K 19/00; A61K 39/00, 48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT, CHEM ABS see below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
MEDLINE see below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT (C12N-015/62/1C OR C07K-019/1C) AND (ANTIGEN: OR EPITOPE# OR IMMUNOGEN:) AND [(TARGET: OR DIRECT) OR (DIMER: OR MULTIMER: OR OLIGOMER:)]
MEDLINE and CHEM ABS: [(FUSION OR HYBRID OR CHIMAER? OR CHIMER?) 2ON (IMMUNOGEN? OR EPITOPE OR ANTIGEN?)] AND(ADJUVANT OR VACCIN? OR (IMMUNE RESPONSE)) AND TARGET?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	US 5 698 679 A (NATIONAL JEWISH CENTRE FOR IMMUNOLOGY AND RESPIRATORY MEDICINE) 16 December 1997 see column 8 line 11 - column 10 line 23 column 10 lines 4-14	1, 2, 4-11
X	WO 96 40941 A (CONNAUGHT LABORATORIES) 19 December 1996 see page 4 lines 7-35, page 11 line 35 - page 13 line 33	1, 2, 4, 5, 7-11
P, X	Nature, vol. 392, March 1998 pages 408-411 Boyle, J. S. et al. "Enhanced responses to a DNA vaccine encoding a fusion antigen that is directed to sites of immune induction" see whole document	1-14

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 April 1998	-6 MAY 1998
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer **JIM CHAN** Telephone No.: (02) 6283 2340

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00208

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. Biol. Chem. vol. 271, No. 52, 1996 pages 33670-33677. Corthesy, B. et al. "A Pathogen-specific epitope inserted into recombinant secretory immunoglobulin A is immunogenic by the oral route" see Introduction and Discussion	1, 2, 4, 5, 7-11
X	J. Clin. Invest. Vol. 98, no. 9, 1996 pages 2001-2007 Liu, C. et al. "$Fc_\gamma RI$-targeted fusion proteins result in efficient presentation by human monocytes of antigenic and antagonist T cell epitopes". Results page 2003 and Discussion pages 2006-7	1, 2, 4-11
X	Immunotechnology Vol. 2, 1996 pages 85-95 Brumeanu, T.D. et al. "Engineering of doubly antigenized immunoglobulins expressing T and B viral epitopes". see figure 3, Introduction and Discussion	1, 2, 4, 5, 7-11
X	J. Virol. Vol. 69, No. 4, 1995 pages 2357-2365 Baier, G. et al. "Immunogenic targeting of recombinant peptide vaccines to human antigen-presenting cells by chimeric anti-HLA-DR and anti-surface Immunoglobulin D antibody Fab fragments in vitro". see figure 1, column 2 page 2363 paragraph 2	1, 2, 4-11
X	Recombinant Vectors in Vaccine Development. Dev. Biol. Stand. Vol. 82, 1994 pages 215-227 (ed. Brown, F.) Lebens, M. and Holmgren, J. "Muscosal vaccines based on the use of cholera toxin B subunit as immunogen and antigen carrier". see page 22 paragraph 3 - page 24	1, 2, 4, 7-11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 98/00208

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	9640941	AU	61178/96	EP	833929

END OF ANNEX